

## AMENDMENTS

In the specification:

On page 5, please delete paragraph [0016] and substitute therefor:

Figure 1D shows a sequence analysis of NAC: NB-ARC homology. Alignment of the NB-ARC domains of human NAC (amino acids 329-547)(SEQ ID NO:19), CARD4 (amino acids 197-408)(SEQ ID NO:20), and Apaf-1 (amino acids 138-352)(SEQ ID NO:21), and *Caenorhabditis elegans* CED4 (amino acids 154-374)(SEQ ID NO:22). Alignment was conducted using Clustal method (Thompson et al., Nuc. Acids Res. 22:4673-4680 (1994)). Identical and similar residues are shown in black and gray, respectively.

On page 6, please delete paragraph [0017] and substitute therefor:

Figure 1E shows alignment of CARD domain of NAC and other CARD-containing proteins(NAC-CARD, SEQ ID NO:23; Apaf-1, SEQ ID NO:24; CARD4, SEQ ID NO:25; CED4, SEQ ID NO:26; CED3, SEQ ID NO:27; hRAIDD, SEQ ID NO:28; hCaspase-2, SEQ ID NO:29; hCaspase-9, SEQ ID NO:30). Alignment was conducted using Clustal method. Identical and similar residues are shown in black and gray, respectively.

On page 6, please delete paragraph [0019] and substitute therefor:

Figure 3 shows homophilic interactions of CARD domains detected by yeast two-hybrid method. Yeast cells were co-transformed with plasmids encoding the indicated proteins fused to LexA DNA binding domain (LexA) and proteins fused to B42 transactivation domain (B42). Transformants were replica-plated on leucine-supplemented plates (Leu+) and leucine-deficient plates (Leu-) to assess protein interactions.  $\beta$ -galactosidase activity (LacZ) was measured for each transformant, and were scaled as: absent (-), weak (+/-), detectable (+), strong (++), very strong (+++), and strongest (++++).

On pages 6-7, please delete paragraph [0021] and substitute therefor:

Figure 5 shows that NAC forms complexes with Apaf-1 and CED4. (A) Complex formation with human Apaf-1. 293T cells were transiently transfected with an expression plasmid encoding HA-tagged human Apaf-1 lacking the C-terminal WD repeats [HA-Apaf-1 ([[Δ]] ΔWD)] in the presence (lanes 2 and 3) or absence (lane 1) of a plasmid encoding myc-tagged full-length NAC (myc-NAC). Transfected cells were lysed and subjected to immunoprecipitation (IP) with either a mouse monoclonal antibody to myc (lanes 1 and 3) or a control mouse IgG (lane 2). Proteins from the immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis (WB) using anti-HA antibodies (bottom panel) followed by anti-myc antibodies (top panel). One tenth of the total cell lysates derived from each transfection were loaded directly in the gel as a control (Lysate). (B) Complex formation with *C. elegans* CED4 protein. Identical procedures and conditions described for Apaf-1 in (A) were employed for CED4 interaction studies with NAC.

On page 10, please delete paragraph [0028] and substitute therefor:

In addition to their role in caspase-activation, CARD domains have been implicated in other cellular processes. Some CARD-containing proteins, for example, induce activation of the transcription factor NF-κB. NF-κB activation is induced by many cytokines and plays an important role in cytokine receptor signal transduction mechanisms (DiDonato et al., Nature 388:548-554 (1997)). Moreover, CARD domains are found in some proteins that inhibit rather than activate caspases, such as the IAP (Inhibitor of Apoptosis Protein) family members, cIAP1 and cIAP2 (Rothe et al., Cell 83:1243-1252 (1995)) and oncogenic mutants of the Bcl-10 protein (Willis et al., Cell 96:35-45 (1999)). Also, though caspase activation resulting from CARD domain interactions is often involved in inducing apoptosis, other caspases are primarily involved in proteolytic processing and activation of inflammatory cytokines (such as pro-IL-1 [[Δ]] β and pro-IL-18). Thus, CARD-containing proteins can also be involved in cytokine production, thus regulating immune and inflammatory responses.

On page 19, please delete paragraph [0057] and substitute therefor:

In one embodiment, a NAC-X protein will comprise an NB-ARC domain of NAC, as previously described, and the CARD domain of CARD-X. In another embodiment, a NAC-X protein will comprise the NB-ARC domain and LRR domain of NAC, the CARD domain of CARD-X, and the TIM-Barrel-like domain from either NAC or CARD-X or a chimera from both. In yet another embodiment, NAC-X will comprise the NB-ARC and LRR domains of NAC and the CARD and TIM-Barrel-like domains of CARD-X. For example, invention chimeric proteins can include residues between 1-947 and 1-1078 of NAC $\beta$  (SEQ ID NO:2) or between 1-918 and 1-1048 of NAC $\gamma$  or NAC $\delta$  (SEQ ID NOs:4 and 6, respectively) in chimera with residues between 1-431 and 56-431 of CARD-X (SEQ ID NO:8). A particular invention chimera is termed NAC-X1 a protein, and comprises the following sequences: NAC $\beta$ -X1, residues 1-1078 of NAC and residues 56-431 of CARD-X, having the resultant amino acid sequence listed in SEQ ID NO:10; NAC $\gamma/\delta$ -X1 residues 1-1048 of NAC $\gamma$  or NAC $\delta$  and residues 56-431 of CARD-X, having the resultant amino acid sequence listed in SEQ ID NO:12. The cDNA encoding NAC [[ $\square$ ]]  $\beta$ -X1 comprises cDNA residues 1-3234 of NAC $\gamma$  and 166-1293 of CARD-X, having the resultant sequence listed in SEQ ID NO:9; and the cDNA encoding NAC $\gamma/\delta$ -X1 proteins comprise cDNA residues 1-3144 of NAC $\gamma$  or NAC $\delta$  and 166-1293 of CARD-X, having the resultant sequence listed in SEQ ID NO:11.

On page 22, please delete paragraph [0065] and substitute therefor:

In accordance with another embodiment of the invention, substantially pure nucleic acid molecules, and functional fragments thereof, are provided, which encode invention NACs. Exemplary invention nucleic acid molecules are those comprising substantially the same nucleotide sequence encoding NAC[[ $\square$ ]]  $\beta$  (SEQ ID NO: 1), NAC[[ $\square$ ]]  $\gamma$  (SEQ ID NO: 3), and NAC[[ $\square$ ]]  $\delta$  (SEQ ID NO: 5).

On pages 59-60, please delete paragraph [00176] and substitute therefor:

*In vitro Protein Binding Assays.* NB-ARC, CARDL, and CARDS in pGEX-4T1 were expressed in XL-1 blue *E. coli* cells (Stratagene), and affinity-purified using glutathione (GSH)-sepharose according to known methods, such as those in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley and Sons (1999). For GST pull-down assays, purified CARDL and CARDS GST fusion proteins and GST alone (0.1-0.5  $\mu$ g immobilized on 10-15  $\mu$ l GSH-sepharose beads) were incubated with 1 mg/ml of BSA in 100  $\mu$ l Co-IP buffer [142.4 mM KCl, 5mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.2% NP-40, 1 mM DTT, and 1 mM PMSF] for 30 min. at room temperature. The beads were then incubated with 1  $\mu$ l of rat reticulocyte lysates (TnT-lysate; Promega, Inc.) containing <sup>35</sup>S-labeled, *in vitro* translated CARDL, CARDS, or control protein Skp-1 in 100  $\mu$ l Co-IP buffer supplemented with 0.5 mg/ml BSA for overnight at 4°C. The beads were washed four times in 500  $\mu$ l Co-IP buffer, followed by boiling in 20  $\mu$ l Laemmli-SDS sample buffer. The eluted proteins were analyzed by SDS-PAGE. The bands of SDS-PAGE gels were detected by fluorography.

On pages 60-61, please delete paragraph [00179] and substitute therefor:

*Protein Interaction Studies in Yeast.* EGY48 yeast cells (*Saccharomyces cerevisiae*: MAT $\alpha$ , trp1, ura3, his, leu2::plexApo6-leu2) were transformed with pGilda-CARDL plasmids (his marker) encoding the LexA DNA binding domain fused to: CARD domains of NAC (CARDL) and caspase-9; pro-caspase-8; Apaf-1 without its WD domain; Bcl-XL, Bax and Bcl-2 without transmembrane domains. EGY48 were also transformed with vector pJG4-5 (trp1 marker) encoding the above listed group of proteins and additionally vRas and FADD as target proteins, fused to B42 transactivation domain, and the cells were transformed with a LexA-LacZ reporter plasmid pSH1840 (ura3 marker), as previously described (Durfee et al., 1993; Sato et al., 1995). Sources for cells and plasmids were described previously in U.S. Patent 5,632,994, and in Zervous et al., Cell 72:223-232 (1993); Gyuris et al., Cell 75:791-803 (1993); Golemis et al., In Current Protocols in Molecular Biology (ed. Ausubel et al.; Green Publ.; NY 1994), each of which is incorporated herein by reference. Transformants were replica-plated on Burkholder's minimal medium (BMM) plates supplemented with leucine and 2% glucose as previously

described (Sato et al., Gene 140:291-292 (1994)). Protein-protein interactions were scored by growth of transformants on leucine deficient BMM plates containing 2% galactose and 1% raffinose.

On page 63, please delete paragraph [00184] and substitute therefor:

*Self-Association of NB-ARC domain of NAC.* *In vitro* translated, <sup>35</sup>S-labeled rat reticulocyte lysates (1 [[ȳ]] μl) containing NB-ARC or Skp-1 (used as a control) were incubated with GSH-sepharose beads conjugated with purified GST-NB-ARC or GST alone for GST pull-down assay, resolved on SDS-PAGE and visualized by fluorography as described above. One tenth of input were loaded for NB-ARC or Skp-1 as controls. In this assay, the NB-ARC-containing fragment of NAC demonstrates a strong ability to homodimerize (Figure 4).

On pages 63-64, please delete paragraph [00186] and substitute therefor:

*Protein-Protein Interactions of NAC.* Transient transfection of 293T, a human embryonic kidney fibroblast cell line, were conducted using SuperFect reagents (Qiagen) according to manufacturer's instructions. The cDNA fragments encoding full-length CED4 and the truncated form of Apaf-1 (Apaf-1ΔWD) comprising amino acids 1-420 of the human Apaf-1 protein were amplified by PCR and subcloned into pcDNA3HA at EcoRI and Xho I sites. Expression plasmids encoding catalytically inactive forms of pro-Casp8 [pro-Casp8 (C/A)] was prepared by replacing Cys 377 with an Ala using site-directed mutagenesis and pro-Casp9 [pro-Casp9 (C/A)] has been described previously, Cardone et al., Science 282:1318-1321 (1998)). 293T cells were transiently transfected with an expression plasmid (2 [[ȳ]] μg) encoding HA-tagged human Apaf-1[[ȳ]] ΔWD, CED4, pro-Casp8 (C/A) or C-Terminal Flag-tagged pro-Casp9 (C/A) in the presence or absence of a plasmid (2 [[ȳ]] μg) encoding myc-tagged NAC (encoding amino acid residues 1-1261 and 1306-1473 of SEQ ID NO:2). After 24 hr growth in culture, transfected cells were collected and lysed in Co-IP buffer [142.4 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.1 % NP-40, and 1 mM DTT] supplemented with 12.5 mM β-

glycerolphosphate, 2 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF, and 1X protease inhibitor mix (Boehringer Mannheim). Cell lysates were clarified by microcentrifugation and subjected to immunoprecipitation using either a mouse monoclonal antibody to myc (Santa Cruz Biotechnologies, Inc) or a control mouse IgG. Proteins from the immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblot analysis using anti-HA antibodies followed by anti-myc antibodies using a standard Western blotting procedure and ECL reagents from Amersham-Pharmacia Biotechnologies, Inc. (Krajewski et al., Proc. Natl. Acad. Sci. USA 96:5752-5757 (1999)).